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The Human Prointerleukin 1β Gene Requires DNA Sequences Both Proximal and Distal to the Transcription Start Site for Tissue-Specific Induction

FUMIHIKO SHIRAKAWA,[1]† KAZUYOSHI SAITO,[1] CHRISTOPHER A. BONAGURA,[1] DEBORAH L. GALSON,[2] MATTHEW J. FENTON,[3] ANDREW C. WEBB,[4] AND PHILIP E. AURON[1,5]*

The Center for Blood Research[1] and Division of Hematology-Oncology, Brigham and Women's Hospital, and Department of Medicine, Harvard Medical School,[2] Boston, Massachusetts 02115; Department of Medicine, Boston University School of Medicine, and Evans Department of Clinical Research, Boston, Massachusetts 02118[3]; Department of Biological Sciences, Wellesley College, Wellesley, Massachusetts 02181[4]; and Department of Medicine, Massachusetts General Hospital, Charlestown, Massachusetts 02129 and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115[5]

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In these studies, we have identified DNA sequences and specific protein interactions necessary for transcriptional regulation of the human prointerleukin 1β (proIL-1β) gene. A cell-type-independent lipopolysaccharide (LPS)-responsive enhancer element located between −3757 and −2729 bp upstream from the transcription start site (cap site) consisted of at least six discrete subregions which were essential to the maximal induction by LPS in transfected monocytes. The enhancer also appeared to mediate phorbol myristate acetate induction in monocytes and IL-1 responsiveness in fibroblasts. Deletion and base substitution mutations along with DNA binding studies demonstrated that the enhancer contained a minimum of three functional protein binding sequences, two of which appeared to be important for gene induction. One of the essential proteins which bound to the enhancer was similar or identical to members of the C/EBP family of transcription factors required for both IL-1- and LPS-specific induction of the IL-6 gene (i.e., the NF-IL6 proteins). When ligated to the proIL-1β cap site-proximal region (located between −131 to +12), both the proIL-1β and the simian virus 40 enhancer elements functioned more efficiently in monocytes than in HeLa cells, which are not normally competent for IL-1β expression. When ligated to the murine c-*fos* promoter, however, the proIL-1β enhancer was inducible in phorbol myristate acetate-stimulated HeLa cells, suggesting the existence of a proIL-1β promoter-proximal requirement for tissue specificity.

Interleukin 1β (IL-1β), a 17-kDa cytokine involved in inflammatory and immunological processes, is produced by activated monocytes/macrophages, fibroblasts, endothelial cells, and other cell types. The IL-1 proteins are translated as larger 31-kDa precursors (proIL-1) which are extracellularly processed into smaller 17-kDa forms (mature IL-1) (4, 20). There are two proIL-1 genes (proIL-1α and proIL-1β) coding for distinct proteins which are observed in many different species (7, 18). The two proIL-1 genes are located on human chromosome 2 (11, 30, 48) and show a high conservation of exon/intron structure (7). IL-1 has a variety of biological activities on a wide range of tissues (see reference 13 for a review). For example, IL-1 induces the production of inflammatory mediators, proteolytic enzymes, and other cytokines that are involved in the joint destruction and inflammation of rheumatoid arthritis (29). IL-1 also modifies connective tissue and bone metabolism through the induction and type switch of collagen and the induction of bone resorption (21).

The proIL-1β gene is normally not transcribed in competent cells until activated by stimuli such as lipopolysaccharide (LPS) or phorbol 12-myristate acetate (PMA) or by 17-kDa IL-1β protein (9, 13, 14). In monocytes stimulated with LPS, IL-1β mRNA is rapidly and transiently transcribed in the absence of protein synthesis (15), suggesting the activation of preexisting transcription factors. Our previous studies revealed the importance of promoter sequences immediately upstream of the transcription start site (cap site) (3, 8, 10, 14, 24, 26) in regulating proIL-1β gene expression. Other studies have shown that the specific induction of the gene requires sequences both far upstream of the cap site as well as in the first intervening sequence (3, 5). The far-upstream sequences described by Bensi et al. (5) define a PMA-responsive enhancer sequence located between positions −2982 and −2795. Further deletion of this region by these investigators revealed that removal of sequences either between −2982 and −2929 or between −2877 and −2795 abolished gene induction. In this report, we show that the LPS-responsive region maps to the general location within the gene previously reported for PMA induction by Bensi et al. (5). However, in contrast to the PMA-inducible enhancer, we demonstrate that the LPS-responsive region (located between −3757 and −2729) extends an additional 150 to 775 bp upstream of the PMA-sensitive region and contains discrete cooperative regions which provide tissue-specific expression only in the context of cap site-proximal proIL-1β promoter sequences. At least three distinct nuclear factors are observed to bind to essential regions of this enhancer. Analyses of specific nucleotide substitutions and deletions demonstrate that decreasing the binding efficiency

* Corresponding author.
† Present address: First Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, 807 Japan.

for at least two of these factors results in a corresponding decrease in LPS-dependent gene expression.

MATERIALS AND METHODS

Endotoxin tests. All materials and solutions, including plasmids, phosphate-buffered saline (PBS), media, and fetal bovine serum (FBS) for tissue culture and transfection, were tested for endotoxin by a *Limulus* amebocyte lysate assay (QCL-1000; Whittaker Bioproducts, Inc., Walkersville, Md.). To reduce endotoxin levels, glassware and CsCl were baked and sterile irrigation water (Baxter Healthcare Corp., Deerfield, Ill.) was used for the preparation of all aqueous solutions. Endotoxin contamination of plasmids, PBS, media, and FBS was also monitored by Northern (RNA) dot blot analysis using IL-1β cDNA as a probe.

Cell lines. A human monocytic cell line, THP-1 (ATCC TIB 202), and a murine macrophage cell line, RAW 264.7 (ATCC TIB 71), were carefully maintained in RPMI 1640 medium–25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) with L-glutamine (0.08 endotoxin unit/ml; Whittaker) containing 10% FBS (defined FBS; less than 0.06 endotoxin unit/ml; HyClone Laboratories, Logan, Utah) and 0.5% penicillin-streptomycin. Cells were discarded when they either exhibited morphological changes or lost their LPS-dependent IL-1β mRNA transcription response. The media for THP-1 cells also contained 5×10^{-5} M 2-mercaptoethanol, which was essential for cell maintenance as recommended by the American Type Culture Collection. RAW 264.7 cells were split after being washed with PBS and then incubated for 10 min in PBS containing 20 mM EDTA. HeLa cells (ATCC CCL 2) and human foreskin fibroblasts were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% FBS and penicillin-streptomycin.

Plasmids. Human IL-1β genomic DNA fragments were derived from clone BDC454 (7) and inserted into chloramphenicol acetyltransferase (CAT) gene plasmid vectors pA10CAT3M (3M), pA10CAT3ME (3ME) (31), and *fos* CAT (*fos*) (33). The nomenclature used for the various proIL-1β constructs reflects the nature of inserted fragments. In this nomenclature, letter symbols reflect the specific restriction enzyme used and numerals locate the endpoints of polymerase chain reaction (PCR) primers. For example, P_2T represents a *Pvu*II-*Taq*I fragment, whereas −2987T represents a fragment derived from a PCR primer starting with −2987 at one end and ending with a *Taq*I-digested end at the other. The DNA fragments were generated either by restriction endonuclease digestion or by using PCR primer oligonucleotides to generate deletions or site-directed mutants as described previously (23). The alkaline lysis plasmid preparations (41) were purified twice on CsCl-ethidium bromide gradients. The DNA used for transfection was assayed for supercoiled density by agarose gel electrophoresis. Endotoxin levels of each batch were monitored by the *Limulus* assay, and only DNA preparations containing less than 0.03 endotoxin unit/μg were used.

DNA sequencing. The upstream region of the human IL-1β gene and all novel CAT constructs were sequenced according to the method reported by Kraft et al. (28), in which double-stranded miniprep DNA was directly used as the template for sequence analysis.

Transfection. RAW 264.7 cells (4×10^6 cells per plate), HeLa cells (5×10^5 cells per plate), or human foreskin fibroblasts (10^6 cells per plate) were plated in 100-mm-diameter culture plates 24 h before transfection. Immediately before $CaPO_4$ transfection, the cells were washed with 10 ml of DMEM three times and incubated in 10 ml of DMEM containing 10% FBS and penicillin-streptomycin for 3 or 4 h. The transfection was accomplished using a $CaPO_4$ mammalian cell transfection kit (5 Prime→3 Prime, Inc., Boulder, Colo.). Twenty micrograms of plasmid was gently mixed with 0.5 ml of 2× HEBS (50 mM HEPES [pH 7.05], 1.5 mM Na_2HPO_4, 10 mM KCl, 280 mM NaCl, 12 mM glucose) and 62 μl of 2 M $CaCl_2$ in 1 ml and incubated at room temperature for 20 min in order to generate a fine precipitate, which was added to the cells. The plate was incubated for 5 h and then subjected to a 15% glycerol shock, which was carried out for approximately 2 to 3 min, depending upon cell type. After being washed with 10 ml of RPMI 1640 medium three times, cells were then incubated in RPMI 1640 medium (or DMEM for HeLa cells) containing 10% FBS and penicillin-streptomycin RAW 264.7 cells were incubated for 18 h and then stimulated with 10 μg of LPS (*Escherichia coli* serotype O55:B5; Sigma Chemical Co., St. Louis, Mo.) per ml for an additional 8 h prior to harvest. HeLa cells were incubated in 10 ml of DMEM containing 10% FBS and penicillin-streptomycin for 40 h and then stimulated with 50 ng of PMA per ml for an additional 24 h. Fibroblasts were incubated for 8 h and then stimulated with 0.1 to 10 ng of human recombinant IL-1β (50) per ml for an additional 15 h.

The THP-1 cells were plated at 4×10^5 cells per ml the day before transfection. The cells were washed twice with unsupplemented RPMI 1640 prior to transfection, and 10^7 cells in 1 ml of the same medium were mixed with DEAE-DNA transfection solution prepared as follows: 10 μg of DNA was mixed with 0.5 ml of 1 M Tris-HCl (pH 7.2) and 7.5 ml of unsupplemented RPMI 1640, mixed well, and added to 1 ml of 2-mg/ml DEAE-dextran (~500,000, average molecular weight; Pharmacia LKB, Uppsala, Sweden). Cells were incubated for 20 min at room temperature prior to centrifugation and resuspension into 10 ml of unsupplemented RPMI 1640 containing 1.5 U of heparin sodium (Elkins-Sinn, Inc., Cherry Hill, N.J.). The cells were then immediately centrifuged and washed in unsupplemented medium prior to resuspension in 10 ml of complete medium and incubated at 37°C in 10-cm-diameter tissue culture dishes. After 24 h, cells were stimulated with LPS (*E. coli* O55:B5; 10 μg/ml; Sigma) or PMA (50 ng/ml) and incubated for an additional 24 h before harvesting.

CAT assay. After harvest, cells were washed with 10 ml of PBS twice and resuspended in 150 μl of 0.25 M Tris-HCl (pH 8.0)–0.1% Triton X-100. After three freeze-thaw cycles, cell lysates were heat inactivated at 65°C for 10 min and microcentrifuged at 4°C for 30 min, and supernatant concentrations were measured with Bio-Rad protein assay kit. The CAT assays were carried out by a liquid scintillation method (45), using 100 μg of THP-1, 50 μg of RAW 264.7, and 100 μg of HeLa cell lysates in 150 and 100 μl of a mixture of 2.5 mM chloramphenicol (Sigma) and 0.5 μCi of [^{3}H]acetyl coenzyme A (NEN). CAT activities were evaluated by calculating slopes within a linear range of the response.

Preparation of nuclear extracts. Nuclear extracts were prepared by a method previously reported (12, 37, 44), with modifications. After a 45-min incubation with LPS (10 μg/ml) or 1-h incubation with PMA (50 ng/ml), 1×10^8 to 3×10^8 cells were harvested, washed with PBS, and incubated in 5 ml of buffer A (10 mM HEPES [pH 7.9] at 4°C, 5 mM $MgCl_2$ 10 mM NaCl, 0.3 M sucrose, 0.1 mM EGTA, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) containing 1 μg of each of the protease inhibitors

antipain, aprotinin, chymostatin, leupeptin, and pepstatin A per ml on ice for 5 to 10 min. After centrifugation, the cells were resuspended in 1 ml of buffer A with protease inhibitors and then Dounce homogenized. The homogenate was microcentrifuged for 30 s, and nuclei were resuspended in 0.8 ml of buffer B with protease inhibitors (20 mM HEPES [pH 7.9], 5 mM $MgCl_2$, 300 mM KCl, 0.2 mM EGTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF) and gently rocked on a platform at 4°C for 30 min. After 30 min of microcentrifugation at 4°C, supernatants were dialyzed against 50 volumes of buffer D (20 mM HEPES [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF) at 4°C for 6 h or overnight. After 30 min of microcentrifugation at 4°C, aliquots of supernatants were frozen at −70°C and protein concentrations were measured by using a Bio-Rad protein assay kit.

Preparation of radiolabeled electrophoretic mobility shift assay (EMSA) probes. Probes were labeled by filling in 3′ recessed ends by using DNA polymerase Klenow fragment and all four α-^{32}P-labeled deoxynucleoside triphosphates (dNTPs) at 3,000 Ci/mmol (DuPont-NEN) as described previously (41). Unincorporated dNTPs were removed by use of G-25 or G-50 columns (5 Prime→3 Prime).

EMSA. EMSA was carried out by the previously reported method (44), using 4% polyacrylamide gel in 0.25× TAE buffer (6.67 mM Tris [pH 7.5], 3.3 mM sodium acetate, 1 mM EDTA). Binding reactions were performed in 15 μl of a buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol, 0 to 40 mM NaCl for different probes) containing ^{32}P-end-labeled probe (0.2 ng, 5,000 to 20,000 cpm), 0.2 to 0.8 μg of poly(dI-dC), and 6 μg of nuclear extracts at room temperature for 20 min. The gels were run at 4°C.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned accession number L06808.

RESULTS

Identification of an upstream region required for LPS induction. To identify the sequence requirements for proIL-1β gene transcription, transiently transfected CAT constructs which contained variable lengths of upstream sequence were introduced into monocyte cell lines. We previously showed that LPS was capable of generating at least a 10-fold stimulation of the endogenous gene in intact cells (15). Our earlier reports on proIL-1β gene transcription in transfected human THP-1 monocytic cells suggested that DNA sequences located between −1097 and +384 in the human proIL-1β gene were not capable of supporting a strong transcriptional induction by LPS (3, 8). We have now extended our upstream clones beyond those previously reported (constructs PT, AT, DT, NT, and HT [8]) to include new CAT constructs terminating almost 4 kb upstream of the transcription start site.

In contrast with our earlier studies, which used THP-1 human monocyte cell lines, the murine monocyte cell line RAW 264.7 (38) was used. This cell line was chosen for our studies because we observed that calcium phosphate transfection of these cells does not result in proIL-1β gene transcription and that a 15-kb human proIL-1β gene corresponding to the entire proIL-1β structural gene and flanking sequences (i.e., DNA located between the previously described upstream *Eco*RI site and the downstream *Sal*I site in clone BDC-454 [7]) is regulated in a manner which is indistinguishable from that of the endogenous murine gene

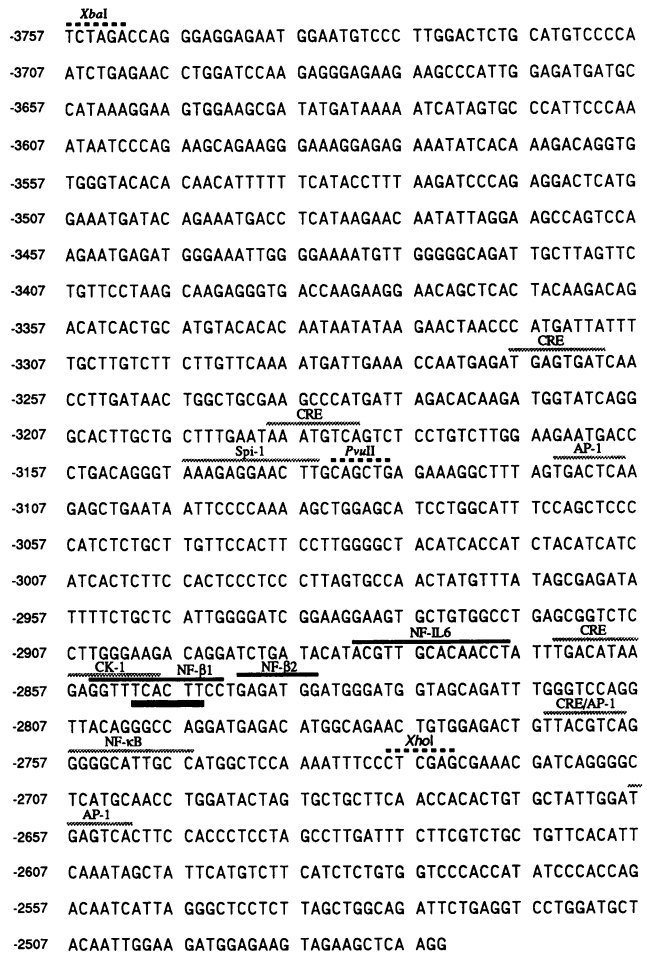

FIG. 1. Upstream nucleic acid sequence for the human proIL-1β gene. The numbering used is aligned with position −2987 in the partial sequence reported by Bensi et al. (5). Specific sequence motifs are indicated by labeled overbars as follows: dotted bars, reference restriction enzyme sites; solid bars, experimentally determined protein binding sites; and gray bars, potential protein binding sites. A bold solid underbar locates the site of a single IRF-1 hexamer motif within the β1 site. Abbreviations for potential site names are as follows: CRE, cyclic AMP response element; CK-1, cytokine gene NF-κB-like factor (43); AP-1, Fos/Jun binding site; and Spi-1, Spi-1/PU.1 binding site. The remaining factors NF-β1, NF-β2, and NF-IL6 are described in the text.

when stably introduced into RAW 264.7 cells (46). Our earlier work suggested a link between the method of THP-1 transfection and gene induction (3, 8). In fact, we have now found that electroporation of these cells resulted in transcriptional induction of the proIL-1β gene and a desensitization from further induction by the addition of a stimulant such as LPS or PMA (data not shown). We have more recently determined that DEAE-dextran can be used to efficiently transfect THP-1 cells without significant proIL-1β gene induction (24, 26). As a consequence, we are now able to examine the specific induction of this gene in the milieu of a human cell line in which we have previously studied the expression of the endogenous gene.

The portion of the human proIL-1β genomic sequence between the *Xba*I site at −3757 and position −2475 is shown in Fig. 1. This sequence extends that which we previously

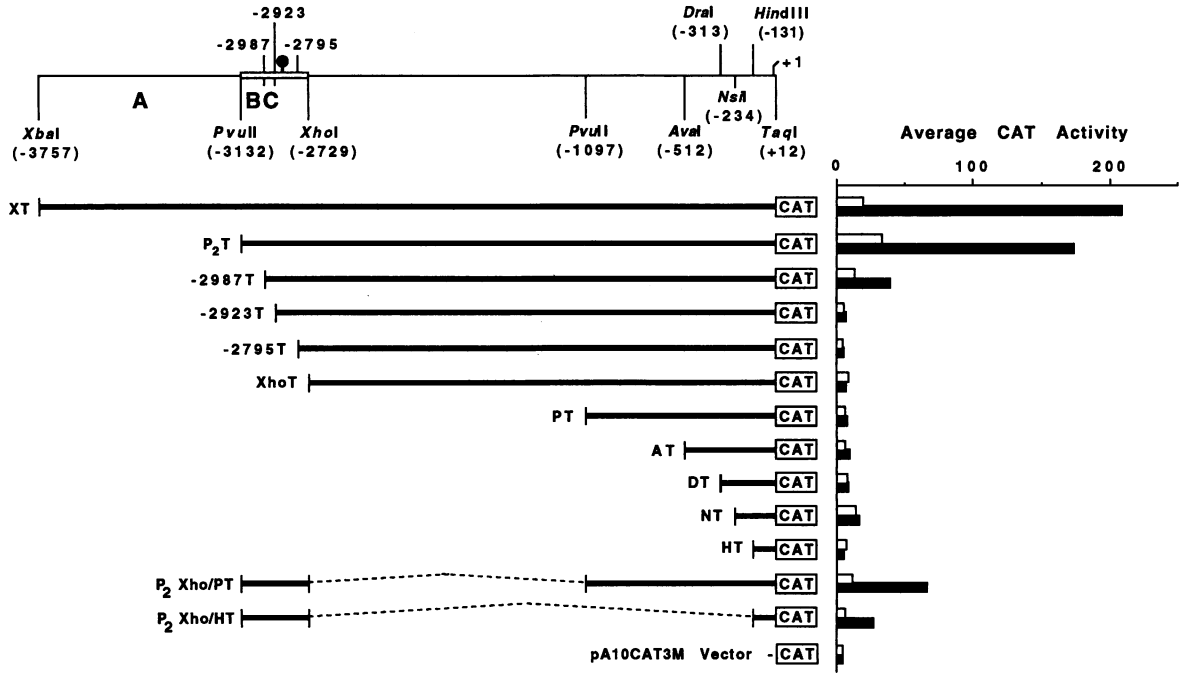

FIG. 2. Summary of CAT data for proIL-1β gene deletions transfected into murine RAW 264.7 cells. Specific proIL-1β gene sequences (horizontal bars) inserted upstream of the CAT coding sequence of the 3M vector are aligned with a schematic locating specific restriction enzyme sites (enzyme names and locations as indicated) and synthetic PCR primers (locations indicated). The schematic also shows the location of the P_2Xho fragment (open box) described in the text and in subsequent figures as well as the location of an NF-IL6-like binding sequence (solid circle). The histogram presents uninduced (open bars) and LPS-induced (solid bars) CAT data represented as the mean of the slopes for plots of time versus counts per minute. The value for each construct represents results from a minimum of two experiments (average was five experiments) with an average deviation of 55%.

reported (7) and contains several potential transcription factor binding site motifs. The sequence data shown in Fig. 1 facilitated the synthesis of oligonucleotide primers which were used to generate some of the CAT constructs shown in Fig. 2, using recombinant PCR synthesis. Specific proIL-1β fragments were subcloned into the promoterless 3M vector and assayed for CAT activity.

The progressive series of upstream end deletions in Fig. 2 shows that an LPS-dependent transcriptional response in murine RAW 264.7 cells was associated with the presence of DNA sequences located between the *Xba*I site at −3757 and position −2923. On the basis of the constructs used in this study, these sequences were delineated into three functional induction-specific regions. The first region, located between −3757 and −3132 (region A), appeared to be responsible for approximately 20% of the LPS-inducible activity, and a second region, between −3132 and −2987 (region B), appeared to correlate with 60% of the activity. The third region, located between −2987 and −2923 (region C), appeared to be responsible for an additional 20% of the activity. Together, these data demonstrated that the −3132 to −2923 sequence (regions B and C) is a necessary component in LPS-specific induction and that the sequence between −3757 and −3132 (region A) was essential for maximal activity. These data do not reveal, however, whether regions B and C are sufficient for induction, since regions downstream of −2923 may be necessary but insufficient for the response.

The upstream LPS induction region reveals internal cooperativity. To address the possible involvement of sequences downstream of −2923 in LPS induction, sequences between −3132 and +12 were further tested by introducing internal deletions downstream of the *Pvu*II site at −2729. The −3132 upstream terminus was chosen because a majority of the LPS induction shown in Fig. 2 mapped between the *Pvu*II site and the *Taq*I site at +12 (construct P_2T). Internal deletions located between the *Xho*I site at −2729 and the functional proIL-1β promoter (8, 24) were constructed in the 3M vector system (constructs P_2Xho/PT and P_2Xho/HT). Transfection of these constructs into RAW 264.7 cells revealed that although downstream sequences between −2729 and −131 might be essential for maximal induction, they were not necessary for the ability to respond to the induction signal (Fig. 2, 3M · P_2Xho/PT and 3M · P_2Xho/HT). In support of this conclusion, when the *Pvu*II-to-*Xho*I (P_2Xho) fragment was tested independently of the proIL-1β promoter-proximal DNA in RAW 264.7 cells by the use of a murine c-*fos* promoter, the level of LPS induction was similar to that of construct 3M · XT (compare 3M · XT with P_2Xho/*fos* in the data for RAW 264.7 cells presented in Table 1).

The P_2Xho/*fos* CAT construct was used to derive a series of additional constructs designed to further characterize the proIL-1β enhancer region. The *fos* CAT vector data in Fig. 3 reveal that the sequence located between −3132 and −2729 (construct 2, P_2Xho) generated a large relative LPS-induced activity when transfected either into RAW 264.7 or THP-1 cells. The presence of additional sequence in construct 1 between −2729 and −2599 (region J in Fig. 3C) yielded a reduced activity which suggested the presence of a negative regulatory element. The sequence between −3132 and −2987 (region B) in the context of the P_2Xho/*fos* CAT construct appeared to be essential (compare constructs 2 and

TABLE 1. Summary of CAT data for proIL-1β constructs transfected into THP-1 and RAW 264.7 cells

Cell line transfected	CAT construct	ProIL-1β DNA positions	CAT activity[a]	
			Uninduced	LPS induced
RAW 264.7	P_2Xho/*fos*	−3132 to −2729	46 ± 28 (10)	280 ± 70 (10)
	3M · XT	−3757 to +12	19 ± 9 (5)	209 ± 38 (5)
THP-1	P_2Xho/*fos*	−3132 to −2729	9 ± 6 (5)	79 ± 38 (5)
	3M · P_2T	−3132 to +12	9 ± 2 (2)	10 ± 2 (2)
	3M · P_2Xho/PT	−3132 to −2729 −1097 to +12	14 ± 4 (3)	16 ± 4 (3)

[a] Represented as the mean of the slopes for plots of time versus counts per minute ± standard deviation. Numbers in parentheses represent the number of experiments performed.

3 with construct 8), in agreement with the upstream deletion data shown in Fig. 2. However, the importance of this region was not as great when sequences between −2780 to −2599 (region I+J) were present (compare construct 2 with constructs 7 and 8), thus suggesting that region I+J can functionally compensate in part for the absence of region B. Furthermore, as demonstrated in Fig. 3, the DNA sequence located between −2894 and −2729 (regions E through I) was important for full LPS-dependent activity (constructs 3 through 6), and the sequence between −2894 and −2864 (region E) appeared to be a significant functional component of this region. The importance of this 30-bp-long sequence was further substantiated by an internal deletion (construct 10). However, although necessary, region E was not sufficient for LPS-dependent activity (in particular, see construct 9). Comparison of the deleted sequence in construct 10 with sequences of a collection of known transcription factor binding sites revealed a close match (12 of 14 bp between −2882 and −2869) with that required for binding of factor NF-IL6, a key component in IL-6 gene transcription (2). Therefore, either NF-IL6 or a related factor may be involved in proIL-1β gene transcription.

As shown in Fig. 2, the P_2Xho/HT and P_2Xho/PT promoter CAT constructs which possessed internal deletions between −2729 and either −1097 or −131 appeared to be only partially active with respect to the intact P_2T construct. This finding suggests that additional sequences between −2729 and −1097 may be necessary to support transcription from the proIL-1β promoter. The data presented in Fig. 3B support and extend those presented in Fig. 2 by defining additional sequence between −2894 and −2729 (regions E through I) to be indispensable for maximal LPS induction of the human proIL-1β gene. It should be noted that the deletion studies shown in Fig. 3 are insufficient to address the importance of DNA-protein binding sites which either are located between −2987 and −2894 or span across the construct terminus located at −2987. However, the deletion data for the proIL-1β promoter (Fig. 2) support the importance of the sequence between −2987 and −2923 (region C). Taken together, Fig. 2 and 3B reveal a complex collection of regions required for LPS induction, as summarized in Fig. 3C.

The data in Table 1 and Fig. 3 show that murine RAW 264.7 cells and human THP-1 cells transfected with the *fos* CAT constructs revealed similar, but not identical, results which may reflect differences between the two systems. The most striking difference is the higher activity, relative to P_2Xho/*fos*, for the P_2-2864/*fos* construct transfected in RAW 264.7 versus THP-1 cells (Fig. 3B, construct 5). In this case, it is interesting to note that the uninduced CAT activity for this construct is threefold greater than that of P_2Xho/*fos*, suggesting that the increased activity may be due to the removal of sequences which act as a silencer only in the RAW 264.7 cells. As described above, Table 1 demonstrates that the P_2Xho/*fos* construct shown in Fig. 3B generated transcription activity in RAW 264.7 cells comparable to that of construct 3M · XT, which contains sequences between −3757 and +12. Table 1 also shows that the relative induction capacity of the P_2Xho/*fos* CAT construct transfected into human THP-1 monocyte cells was similar to that of the transfected murine cells. Thus, it is likely that the requirements for LPS-inducible transcription are common to human and mouse cells, reflecting the likely involvement of similar transcription factors. One major difference between the RAW 264.7 and THP-1 cells, however, was a strong suppression of activity in THP-1 associated with the presence of sequences located downstream of the P_2Xho fragment (i.e., the low activity shown in Table 1 for 3M · P_2T and 3M · P_2Xho/PT in THP-1 cells). The presence of suppressive sequences suggests the possible involvement of additional positive-acting factors in THP-1 cells which may require additional sequences that lie beyond the boundaries of the P_2T DNA. Antisuppressive factors could bind to such sequences and overcome the suppression in the intact chromosomal gene.

Identification of protein binding sites within the proIL-1β LPS response element. EMSAs were used to examine the nature of specific DNA-protein binding within the P_2Xho enhancer. Figure 4A is a schematic representation of regions D through H indicating the locations of gene fragments which were used either as radiolabeled EMSA probes or as specific unlabeled competitors. Figure 4B shows the sequences of the synthetic double-stranded oligonucleotides which were used for EMSA analysis. The results shown in Fig. 5A demonstrate that oligonucleotide IL6/I, containing the NF-IL6-like sequence in the human proIL-1β gene, did bind a THP-1 cell nuclear factor which was effectively competed for by unlabeled oligonucleotide IL6, corresponding to the human NF-IL6 binding site previously characterized by Akira et al. (2) (compare lanes 1 through 3 with lanes 6 and 7). As indicated in Fig. 5, this factor generates a pattern of two or more bands, similar to that reported for the various forms of the NF-IL6 (C/EBP) factors (6, 27, 49). The binding of this protein was also specifically competed for by the unlabeled oligonucleotide IL6/I (lanes 4 and 5) and was not strongly competed for by mutated versions of the IL6/I binding sequence (ΔIL6/I and IL6/Im; lanes 8 through 11). Figure 5B shows that the two distinct mutated radiolabeled IL6/I probes did not bind the THP-1 NF-IL6-like protein as well as they bound the wild-type sequence found in the proIL-1β gene. In particular, the ΔIL6/I probe appeared to bind only a nonspecific protein which could not be displaced

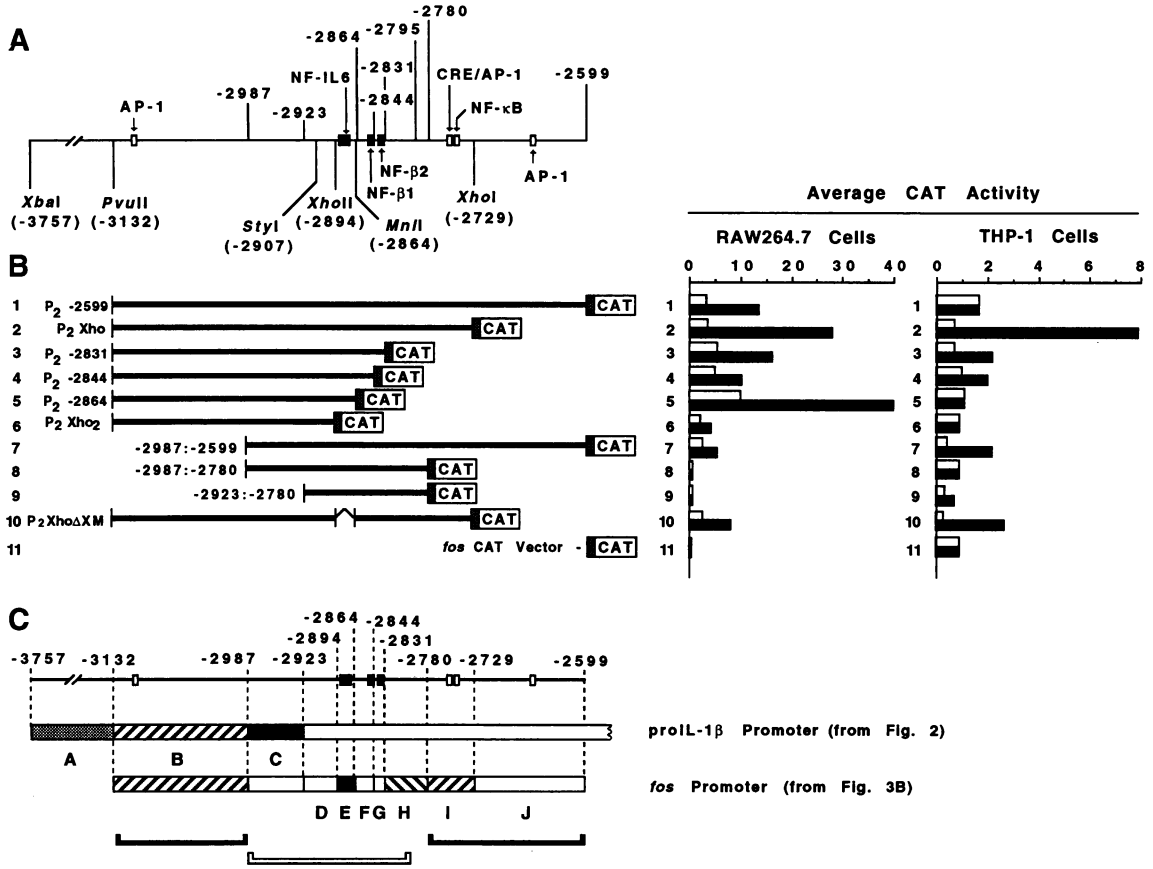

FIG. 3. Functional characterization of the human proIL-1β far-upstream LPS-responsive region. (A) Schematic map of the sequence located between −3757 and −2599. Open boxes locate sequence motifs suggesting potential protein binding sites. Closed boxes locate experimentally determined protein binding sites. (B) Deletion constructs. Deletion studies for the −3132 to −2599 region used the murine c-*fos* promoter transfected either into RAW 264.7 or THP-1 cells. The indicated proIL-1β gene sequences were inserted immediately upstream of the −56 to +109 region of the murine c-*fos* promoter (stippled box) in the *fos* CAT vector. This region consists of positions −56 to +109 of the murine c-*fos* genomic DNA sequence and appears to be devoid either of enhancer activity or of a strong relationship to sequence motifs for a large collection of known transcription factors (2a, 33). The CAT data are calculated and presented as for Fig. 2. The value for each construct represents results from a minimum of two experiments (average was five experiments) with average deviations of 29% for the data from RAW 264.7 cells and 52% for the data from THP-1 cells. (C) Summary of the murine RAW 264.7 cell CAT data presented in Fig. 2 and panels A and B. Shaded regions correspond to those that are required (solid), strongly supportive (cross-hatched), weakly supportive (stippled), and not determined or unimportant (open). These regions are designated A through J and are described in detail throughout the text. Bold brackets locate compensatory regions which appear to be able to substitute for one another. The open bracket locates the PMA-responsive region previously reported (5).

by unlabeled IL6/I competitor (compare lane 6 with lanes 9 and 10). In contrast, the IL6/Im probe bound a specific NF-IL6-like displaceable factor (lanes 14 and 15) but did not form all of the NF-IL6-like complexes observed with the IL6/I probe (compare lanes 1 and 12). This result indicated that the sequence alteration did not completely abrogate binding (see also Fig. 5A, lane 11). It therefore appeared that NF-IL6, or another member of the C/EBP protein family, binds to an essential LPS-responsive region of the human proIL-1β gene. A counterpart to the THP-1 cell NF-IL6-like factor appeared to also exist in RAW 264.7 cells (not shown). In contrast to THP-1 cells, RAW 264.7 cells did not generate high-quality EMSA data but rather yielded band patterns which, although similar to that observed with THP-1 extracts, were more diffuse.

Figure 6A shows EMSA analysis of the region immediately downstream of the NF-IL6-like binding site, using an 84-bp oligonucleotide probe (probe M1 in Fig. 4A). Two primary groups of bands, labeled NF-β1 and NF-β2, appeared to define the binding of two protein classes. The competitor oligonucleotides MSS and 23D appeared to displace both the NF-β1 and NF-β2 band groups, whereas oligonucleotides D8, β1, and β2 selectively displaced only one group or the other. On the basis of these data, it appears as though at least two proteins bind to the two adjacent sites on the M1 oligonucleotide probe. Attempts to footprint these two binding sites on oligonucleotide M1 by using methylation interference have generated very weak results, probably because of the complex band patterns for the two groups of bands. Nonetheless, the methylation interference data suggested the possibility that sites β1 and β2 overlap at positions −2845 and −2844 (data not shown) and have allowed us to design mutant oligonucleotides (Fig. 4B) which decreased the binding of these factors. Figure 6A, lanes 12 and 13 as well as lanes 17 and 18, reveal that two such mutants (β1m and β2m), in contrast to the wild-type sequences (β1 in lanes 10 and 11; β2 in lanes 15 and 16), were not efficient competitors for the NF-β1 and NF-β2 factors. The locations

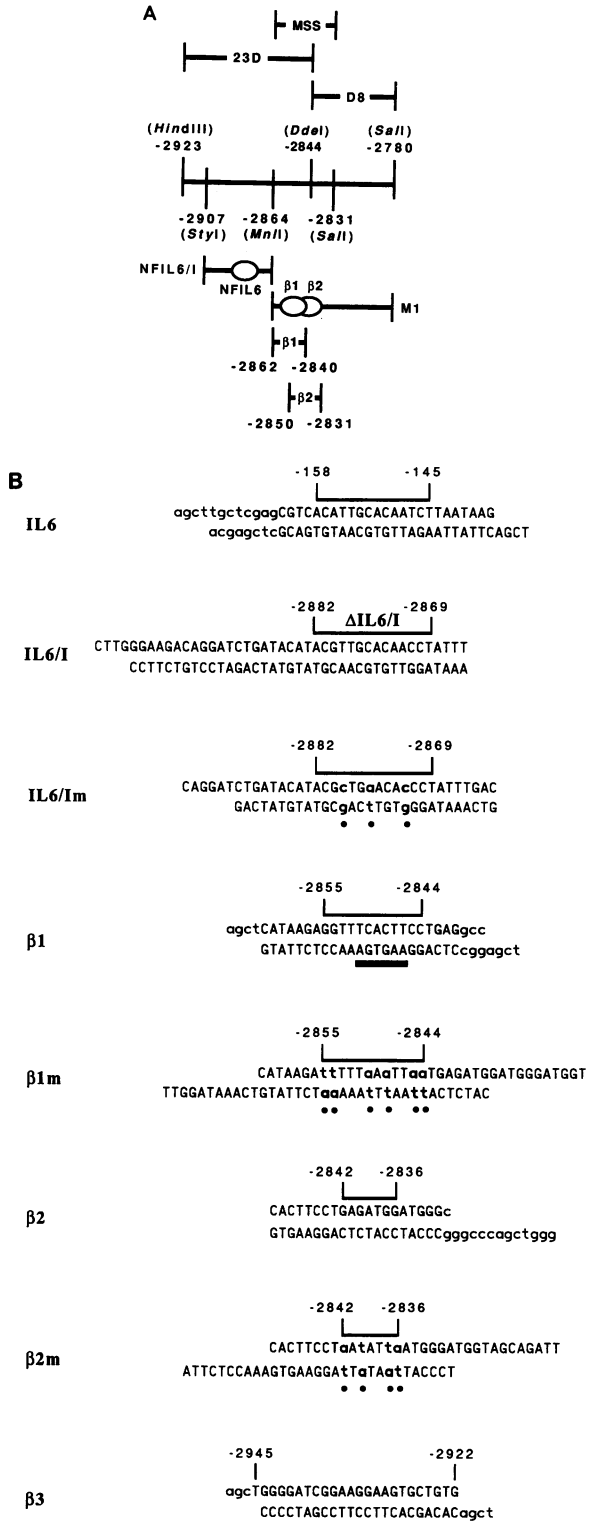

FIG. 4. Gene fragments and oligonucleotides used for EMSAs. (A) Schematic map showing the location relative to the proIL-1β gene sequence for various fragments used either as EMSA probes or as competitors (see text for details). Open ovals locate factor binding sites as described in the text. (B) Sequences for synthetic double-stranded oligonucleotides used as EMSA probes or competitors. Uppercase letters denote native proIL-1β sequence, whereas lowercase letters represent nucleotides not found in proIL-1β. The IL6 probe contains the human IL-6 gene binding site for the NF-IL6 factor as described previously (2), whereas IL6/I contains the human proIL-1β NF-IL6-like sequence. The ΔIL6/I designation indicates the region deleted in the ΔIL6/I deletion oligonucleotide. Bracketed regions above sequences signify specific protein binding and recognition sites defined by the location of mutated nucleotides which affect factor binding. The bold bar beneath the β1 oligonucleotide sequence denotes a single copy of the beta interferon IRF-1 hexamer motif. Bullets indicate the sites of specific mutations designed to abrogate protein binding. Nucleotide substitutions in the IL6/Im probe were derived from the studies of Akira et al. (2). Substitutions in the β1m and β2m probes were derived from preliminary methylation interference analysis results (see text).

of the base changes in the two mutant oligonucleotides (Fig. 4B) define the binding sites for these two factors. Therefore, in good agreement with our tentative footprint data, the binding sites for NF-β1 and NF-β2 appear to be separated by no more than a single base pair.

To more carefully examine the nature of the NF-β1 and NF-β2 binding sites, the β1 and β2 oligonucleotides used as unlabeled competitors in Fig. 6A were employed as radiolabeled EMSA probes (Fig. 6B and C). These data, which were derived by using probes substantially shorter than the M1 oligonucleotide used in Fig. 6A, suggested some interesting contrasts. First, the overall binding complexity was reduced for both factors, and in particular, the NF-β2 factor appeared to be represented as a single specific band rather than three bands (Fig. 6B). In addition, the binding of factor NF-β1 appeared to be compromised by the use of the shorter β1 oligonucleotide, suggesting a requirement either for additional DNA sequence or, perhaps, the binding of NF-β2 (Fig. 6C).

Mutant protein binding sites in P_2Xho/*fos* CAT decrease LPS-specific enhancer activity. To ascertain the functional importance of specific protein-DNA associations, the above-described mutations were examined for functional relevance in the context of the LPS-responsive P_2Xho/*fos* CAT construct. Four distinct mutants were derived from overlapping PCR primer techniques as described previously (23). Each of the mutants contained a single mutated binding site (i.e., IL6/Im, ΔIL6/I, β1m, and β2m) which contained either multiple base substitutions or a deletion in P_2Xho/*fos* CAT. The resulting plasmids were transfected into RAW 264.7 cells and assayed for CAT activity as shown in Table 2. These data suggested that the NF-IL6 and NF-β1 binding sites were important for LPS-induced gene transcription. In contrast, the presence of the β2 site did not appear to be required. In addition, Table 2 shows that the IL-6 site is also important for expression in THP-1 cells. Since the 14-bp deletion in P_2XhoΔIL6/I (Table 2) and the longer 31-bp deletion in P_2XhoΔXM (Fig. 3B, construct 10) generated similar LPS-induced CAT activities relative to the P_2Xho/*fos* CAT construct, it is likely that the loss of the IL-6 site is responsible for the reduced activity of P_2XhoΔXM.

Induction of the human proIL-1β gene by PMA and mature IL-1β protein. The LPS induction of IL-1 provides a useful model system for monocyte activation, which has been extensively investigated (13). However, other agents such as PMA and mature IL-1 proteins have also been reported to induce proIL-1β gene transcription (9, 16). The PMA stimulus is especially relevant because it has been shown to induce expression via a distinct pathway (16, 42). In addition, the potential for autocrine effect renders the action of mature IL-1β on its own gene of interest as well. Transient transfection of primary human dermal fibroblasts with con-

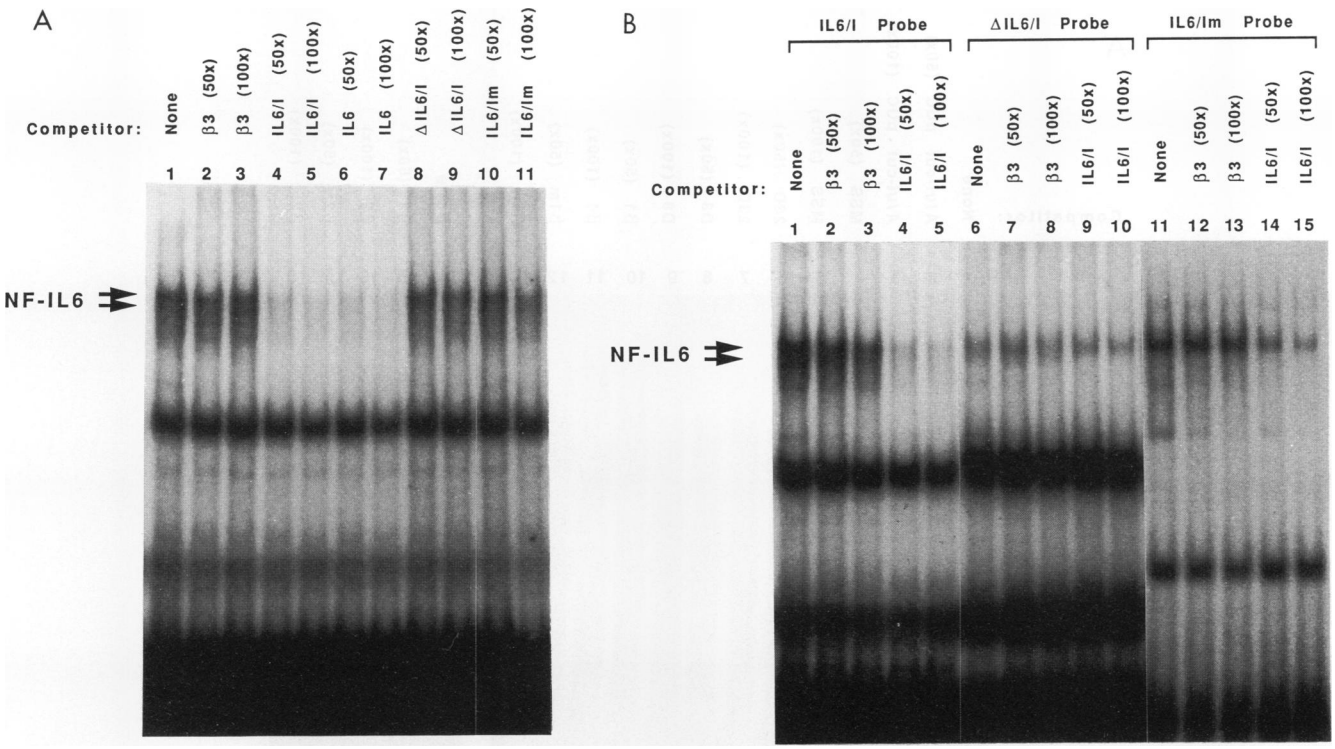

FIG. 5. Binding of an NF-IL6-like protein to the proIL-1β gene. All lanes contained nuclear extract derived from THP-1 cells treated with LPS (10 μg/ml) for 45 min. Unlabeled competitors indicated above each lane were used in molar excess, as indicated by the figures within parentheses. The cold competitor labeled as β3 corresponds to DNA sequence located between −2945 and −2922, which appears to be unrelated to either the NF-β1 or NF-β2 sequence. (A) EMSA using radiolabeled probe IL6/I; (B) EMSA using radiolabeled probes and unlabeled competitors as indicated and LPS-treated THP-1 nuclear extracts.

structs 3M · P_2T and 3M · XT was inducible with recombinant human IL-1β protein (Table 3). Furthermore, the IL-1β protein induction of 3M · XT appeared to respond in a dose-dependent fashion. Similarly, Table 3 also shows that transfection of both RAW 264.7 monocytes and HeLa cells with the P_2Xho/*fos* construct was sensitive to PMA as a stimulus. Consequently, the P_2Xho region of the proIL-1β gene appears to respond to at least two distinct induction signals, namely, LPS and PMA, and may be involved in the response to mature IL-1β protein as well.

Cell type transcription specificity requires sequences close to the transcription start site. Since normal cellular expression of the proIL-1β gene is tissue specific, in that only certain cell types (e.g., monocytes, fibroblasts, endothelial cells, and smooth muscle cells) are induction competent in the absence of other differentiation or activation signals such as viral infection, it is of interest to address the question of whether there might be a region or regions essential for tissue-specific expression. We chose to use HeLa cells, which we had previously determined were refractory to proIL-1β induction by PMA (8), for transfection studies aimed at examining cell specificity. As can be seen in Table 3, the P_2Xho/HT construct containing the proIL-1β promoter was 37-fold less efficiently transcribed in uninduced HeLa than was the P_2Xho/*fos* construct. In addition, P_2Xho/*fos* is PMA inducible in HeLa cells, but P_2Xho/HT is not. Examining these efficiency differences further, we found that P_2Xho/HT was also eightfold more efficiently expressed in LPS-treated murine RAW cells than in PMA-stimulated HeLa cells (Table 4). This finding contrasted with results for the P_2Xho/*fos* construct, which was efficiently induced in both cell lines. To further examine this phenomenon, the proIL-1β promoter sequences were introduced into the promoterless 3ME vector, which contains one complete copy of the 232-bp simian virus 40 (SV40) enhancer. Table 4 reveals that in the context of the SV40 enhancer, which is generally not tissue specific (39), the proIL-1β promoter (construct 3ME · HT) appeared to be almost 30-fold more efficient at supporting induced transcription in RAW monocytes than in HeLa cells. On the other hand, pSV2CAT, in which the SV40 enhancer was used in the context of the SV40 early promoter, was more highly induced in HeLa than in RAW cells. In addition, while pSV2CAT was almost 3-fold more highly expressed than 3ME · HT in RAW cells, in HeLa cells the difference was more than 200-fold. These data suggest that proIL-1β gene tissue specificity appears to be dependent upon sequences close to the transcription start site and independent of the nature of the enhancer.

DISCUSSION

In this study, we have identified sequences within the human proIL-1β gene which are essential for the induction and maintenance of transcription. Transient transfection of murine RAW 264.7 cells by using a CAT gene reporter demonstrates that far-upstream sequences located between −3132 and −2729 (P_2Xho fragment) are important for the induction response. On the basis of CAT activity, a minimum of two subregions (labeled C and E in Fig. 3C) contained within the P_2Xho fragment are essential for activity. These two subregions appear to be required for activity and cooperate with adjacent upstream sequences in regions

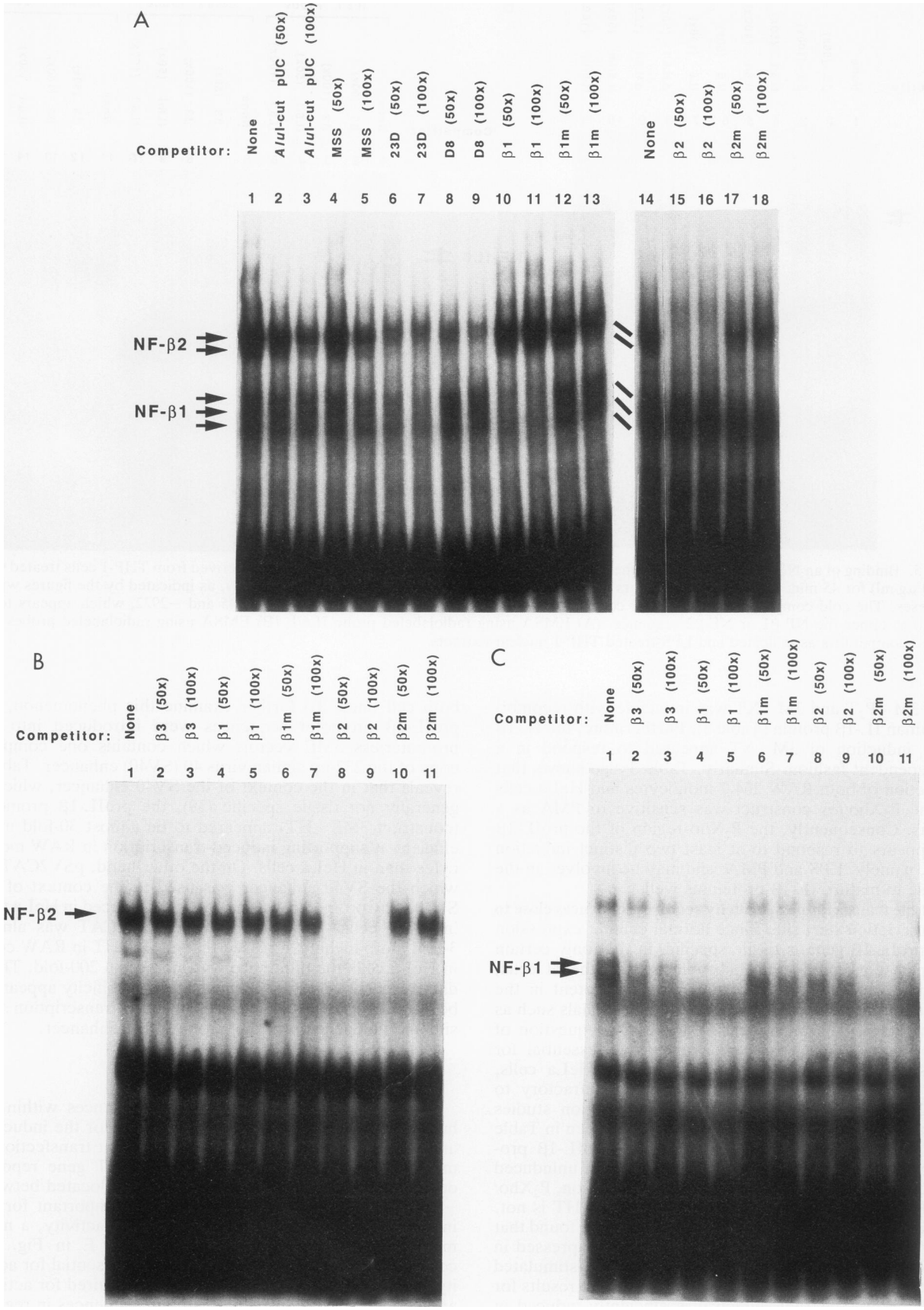
A
Competitor:
None
AluI-cut pUC (50x)
AluI-cut pUC (100x)
MSS (50x)
MSS (100x)
23D (50x)
23D (100x)
D8 (50x)
D8 (100x)
β1 (50x)
β1 (100x)
β1m (50x)
β1m (100x)
None
β2 (50x)
β2 (100x)
β2m (50x)
β2m (100x)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
NF-β2
NF-β1
B
Competitor:
None
β3 (50x)
β3 (100x)
β1 (50x)
β1 (100x)
β1m (50x)
β1m (100x)
β2 (50x)
β2 (100x)
β2m (50x)
β2m (100x)
1 2 3 4 5 6 7 8 9 10 11
NF-β2
C
Competitor:
None
β3 (50x)
β3 (100x)
β1 (50x)
β1 (100x)
β1m (50x)
β1m (100x)
β2 (50x)
β2 (100x)
β2m (50x)
β2m (100x)
1 2 3 4 5 6 7 8 9 10 11
NF-β1

TABLE 2. Summary of CAT data for mutated P_2Xho/*fos* CAT proIL-1β constructs

Cell line transfected	*fos* CAT construct	Binding site mutated	CAT activity[a]		Relative LPS induced CAT (%)
			Uninduced	LPS induced	
RAW 264.7	P_2Xho	None	46 ± 28 (10)	280 ± 70 (10)	100
	P_2Xho IL6/Im	IL6	18 ± 9 (4)	98 ± 68 (4)	35
	P_2XhoΔIL6/I	IL6	15 ± 8 (5)	127 ± 34 (5)	45
	P_2Xho β1m	β1	15 ± 6 (4)	125 ± 32 (4)	45
	P_2Xho β2m	β2	45 ± 7 (2)	313 ± 170 (2)	112
	fos CAT control		4 ± 1 (6)	4 ± 2 (6)	1.4
THP-1	P_2Xho	None	9 ± 6 (5)	79 ± 38 (5)	100
	P_2XhoΔIL6/I	IL6	6 (1)	24 ± 7 (2)	30
	fos CAT control		9 ± 9 (8)	9 ± 5 (8)	11

[a] Calculated as described in Table 1, footnote *a*.

A and B and with adjacent downstream sequences located in regions F through I to support a maximal response. The P_2Xho-inducible activation region appears to be cell type independent in the context of a non-proIL-1β promoter (murine c-*fos* promoter) but is sensitive to cell type when associated with its cognate promoter. Therefore, this cell type specificity does not reside exclusively within the proIL-1β enhancer region but rather requires the proIL-1β promoter. This promoter can also demonstrate cell specificity when associated with the noncognate SV40 enhancer (Table 4). Thus, the tissue specificity for proIL-1β gene transcription may reside proximal to the promoter between positions −131 and +12. Important regions also exist between −2729 and −1097 as well as between −1097 and −131, as demonstrated by the decreased activity of the two internal deletion constructs (P_2Xho/PT and P_2Xho/HT) shown in Fig. 2.

The transfection data presented here contrast with those previously reported by Bensi et al. (5) in several ways. First, both the THP-1 and the RAW 264.7 cells used in this study, unlike the THP-1 derivative cell line described by Bensi et al., exhibit endogenous proIL-1β mRNA synthesis in response to treatment with LPS. Therefore, in contrast to the previous report, we have demonstrated that transfected constructs are LPS responsive. The LPS-responsive region reported here (P_2Xho plus the additional upstream region A) extends as much as 775 bp farther upstream than does the previously reported PMA-responsive region. Furthermore, the LPS-specific transcriptional activity for a construct containing the PMA-responsive region of Bensi et al. but lacking the additional upstream sequences reported here (Fig. 2, construct −2987T) is only 20% of that observed when the additional sequences are present (Fig. 2, construct XT). Finally, the earlier study failed to note the functional segmentation of the region and the apparent cooperativity of these multiple elements as well as the requirement for the cap site-proximal sequences in establishing tissue specificity or maximal activity. It is possible that some of these differences relate to distinctions among the cell types used, the nature of the induction agents, and the conditions used to maintain the cells. For example, as mentioned above, we have carefully monitored endotoxin levels throughout all procedures in order to minimize induction artifacts. Our observation that primary human dermal fibroblasts transiently transfected with constructs 3M · P_2T and 3M · XT respond to induction by IL-1β protein (Table 3) demonstrates that the P_2Xho fragment contains a target for the activation of transcription by LPS and PMA and possibly also IL-1β protein.

Table 1 shows that in THP-1 cells, the response of the P_2Xho/*fos* CAT construct is similar to that observed in the murine RAW 264.7 cells. In contrast, the 3M · P_2T and 3M · P_2Xho/PT CAT constructs are not inducible in THP-1 cells, whereas they are inducible in RAW 264.7 cells (Table 1 and Fig. 2). These data suggest the possible involvement of a transcriptional silencer in THP-1 cells located between −1097 and +12 along with the potential activation sites. This is true both for constitutively expressing electroporated cells and for the inducible DEAE-dextran-transfected cells (not shown). The earlier observation that a THP-1 silencer activity may be overcome by the presence of the first intervening sequence in the proIL-1β gene (3, 5) suggests that additional sequences may be essential to completely reconstitute normal gene function in these cells. The human fibroblast studies (Table 3) contrast with the THP-1 transfection results shown in Table 1 in that 3M · P_2T and 3M · XT generated a substantially greater induction, suggesting that either cell type or stimulus (i.e., LPS versus mature IL-1β protein) may affect the degree to which the effect of the downstream suppressive region is counteracted.

The P_2Xho fragment contains a recognition site which resembles that of the human NF-IL6 factor (2), which has been identified as murine C/EBPδ (6) and IL-6DBP in the rat (36). The human NF-IL6 factor has been shown to be composed of at least two C/EBP-like proteins (C/EBPβ and C/EBPγ), which can form homo- and heterodimers (6, 27, 49). At least one of the NF-IL6 proteins has been shown to form a complex with the p50 subunit of NF-κB (32). The NF-IL6 factors have been postulated to be responsible for IL-1, IL-6, tumor necrosis factor, and LPS activation of the IL-6 gene (1). The binding site for this factor in the proIL-1β gene is located within the functionally essential region E. An

FIG. 6. EMSA using radiolabeled probes M1, β1, and β2 (depicted in Fig. 4). Nuclear extracts were derived from THP-1 cells treated with LPS (10 μg/ml) for 45 min. Unlabeled competitors shown above each lane were used in molar excess, as indicated by the figures within parentheses. The *Alu*I-cut pUC nonspecific competitor used in lanes 2 and 3 represents an equimolar mixture of five fragments ranging between 136 and 93 bp in length derived by preparative agarose gel electrophoresis of a complete *Alu*I digest of plasmid pUC19. The cold competitor labeled β3 corresponds to DNA sequence located between −2945 and −2922, which appears to be unrelated to either the NFβ1 or NFβ2 sequence (see Fig. 4). (A) Probe M1; (B) probe β2; (C) probe β1.

TABLE 3. CAT data for PMA and IL-1β protein induction of transfected proIL-1β constructs

Cell type transfected	CAT construct	Inducer (concn used [ng/ml])	CAT activity[a]	
			Uninduced	Induced
Primary fibroblast	3M · P_2T	IL-1β (0.1)	7 ± 3 (4)	15 ± 8 (4)
	3M · XT	IL-1β (0.1)	4 (1)	11 (1)
	3M · XT	IL-1β (1)	4 (1)	25 (1)
	3M · XT	IL-1β (10)	4 (1)	40 (1)
RAW 264.7	P_2Xho/*fos*	PMA (200)	22 (1)	58 (1)
	P_2Xho/*fos*	PMA (1,000)	22 (1)	78 (1)
HeLa	P_2Xho/*fos*	PMA (50)	372 ± 95 (3)	2,005 ± 123 (3)
	P_2Xho/HT	PMA (50)	10 ± 0 (2)	10 ± 4 (2)

[a] Calculated as described in Table 1, footnote *a*.

EMSA using DNA from this region reveals that the binding to the IL-1 sequence is indistinguishable from that observed with use of the reported NF-IL6 oligonucleotide (Fig. 5). The band shift observed in the EMSA using the IL-1 oligonucleotide not only is similar to that reported elsewhere (2, 25) but also is competed for by the bona fide NF-IL6 oligonucleotide. Therefore, it is likely that an NF-IL6-related factor is indeed binding to this essential region. The P_2Xho/*fos* CAT plasmid mutants, containing either the NF-IL6/Im base pair substitutions which reduce the binding of this factor or a deletion of the entire NF-IL6 binding site, show a 55 to 65% decrease in LPS-inducible activity in RAW 264.7 cells and a 79% decrease in THP-1 cells. This finding demonstrates that the NF-IL6 factor has a functional role. The fact that the activity is not completely abrogated by the NF-IL6 binding site deletion suggests a strong but partial role in activity.

We have observed that the NF-IL6 factor is present in significant amounts prior to LPS treatment and that the kinetics of the LPS-dependent increase in NF-IL6 factor binding in THP-1 cell nuclear extracts do not correlate with our earlier reported (15) rate of transcriptional induction (not shown). The presence of significant amounts of this factor prior to induction and the inactivity of a construct containing the NF-IL6 binding site (construct 9 in Fig. 3B) suggest that other cooperating factors are involved. It should be noted that the expression of this factor by unstimulated cells is not inconsistent with an involvement in a critical role in LPS induction, since the NF-IL6 factor could require posttranslational modification or the association with other protein factors. The involvement of NF-IL6 in the transcriptional activity of proIL-1β broadens the range for this factor to yet another gene involved in the manifestation of the acute-phase response which alters an organism's physiology in response to inflammatory stress.

The other two factors which bind to the P_2Xho fragment include NF-β1, which binds to a sequence resembling a binding site for factor IRF-1, and the immediately adjacent novel NF-β2 binding site. Factor IRF-1 has been reported to be essential for induction of the human beta interferon gene, which can be induced by virus, IL-1, tumor necrosis factor, platelet-derived growth factor, and colony-stimulating factor 1 (17, 22). This factor, which was originally defined in terms of its binding half-sequence (the hexamer motif) (17), has recently been characterized (34) and has been shown to be induced by stimuli such as lectins and LPS as well as by various cytokines (22). The fact that NF-β1 appears to bind to a region containing a single hexamer motif (i.e., an IRF-1 half-site; see Fig. 1) suggests the possibility that NF-β1 consists of an IRF-1-related protein heterodimer. The modification of the β1m site appears to have a negative effect on activity, whereas the β2m mutation results in a slight activity increase (Table 2). Two downstream deletion constructs shown in Fig. 3B (constructs 3 and 4) support the minimal importance of the β2 site relative to that of β1. Like the binding of the NF-IL6 factor, the bindings of NF-β1 and NF-β2 are not significantly affected by stimulation (not shown).

The mutation and deletion data presented here support a preliminary model in which NF-IL6 binds to region E and additional factors bind to regions A through C as well as to regions F through I. These factors are each necessary, but

TABLE 4. Cell specificity of transfected human proIL-1β gene sequences

CAT construct	Promoter	Enhancer	Induced CAT[a]		Fold CAT over control	
			RAW 264.7 (LPS)	HeLa (PMA)	RAW 264.7 (LPS)	HeLa (PMA)
Test plasmids						
P_2Xho/*fos*	c-*fos*	proIL1β	280 ± 70 (10)	2,005 ± 123 (3)	76	41
3M · P_2Xho/HT	proIL1β[b]	proIL1β[b]	27 ± 9 (3)	9.7 ± 4.1 (2)	8	1.0
3ME · HT	proIL1β[b]	SV40	79 (1)	11 (1)	20	0.7
pSV2CAT	SV40 early	SV40	216 (1)	2,346 ± 789 (3)[c]	54	147
Controls						
fos vector	c-*fos*	None	3.7 ± 0.1 (2)	49 ± 5.8 (2)		
3M vector	None	None	3.5 ± 1.6 (6)	10 ± 0.7 (2)		
3ME vector	None	SV40	4.0 (1)	16 (1)		

[a] Calculated as described in Table 1, footnote *a*. RAW 264.7 cells were transfected with $CaPO_4$, and HeLa cells were transfected with DEAE-dextran. The *fos* and 3M vectors were used as controls for P_2Xho/*fos* and 3M · P_2Xho/HT, respectively. The 3ME vector contains the SV40 enhancer sequence and was used as a control for the normalization of both the 3ME · HT and the pSV2CAT data.

[b] The proIL1β promoter and enhancer used sequences located between −131 to +12 and −3132 to −2729, respectively.

[c] Unstimulated value (i.e., no PMA added).

insufficient, for gene induction. Preliminary EMSA analysis suggests that at least three additional factors bind to the B through C region (not shown). Such interdependence among discrete factors is not novel and has been described for other transcription systems. In particular, Drouet et al. (13a) have shown that three of four cooperating NF-κB sites must be eliminated in order to significantly decrease the LPS induction of the murine tumor necrosis factor alpha gene. Therefore, the mutation of a single binding site for genes requiring multiple transcription factor associations is not necessarily sufficient for inactivation.

A screening of the distribution of the three DNA-binding factors mentioned above has been initiated by using the EMSA probes described in this study to test for binding in nuclear extracts prepared from IL-1-treated human primary dermal fibroblasts, PMA-treated HeLa cells, and untreated COS cells (40). As a result, it appears as though NF-β2-like factors are abundant in all these cell types, whereas NF-β1 could not be detected in any of them. Like NF-β2, NF-IL6-like factors (as judged by specific binding to an IL6/I probe) are present in all of the cells used, although the abundance in HeLa and COS cells appeared to be significantly less than in fibroblasts. The presence of NF-IL6/I-binding factors in all of these cells is consistent with the expression of the P_2Xho/*fos* construct in HeLa cells (Table 4). The apparent lack of NF-β1-like binding in any of the cells tested suggests that this factor may not be essential for proIL-1β gene induction in IL-1-treated fibroblasts or PMA-treated HeLa cells. The IL-1 transcriptional response profile fits well with induction by other well-known transcription factors such as AP-1, CREB, and NF-κB. A search of the sequence in the vicinity of the upstream regulatory region suggests the presence of several sequence motifs which may serve as binding sites for such factors (Fig. 1). Specific internal deletions at these sites will be required to establish the exact role of each of these factors.

We had previously reported that the proIL-1β gene promoter is contained within 131 residues of the transcription start site (47), is highly conserved between human and mouse sequences (8), and binds a protein present in cells competent for proIL-1β gene transcription (3, 8, 24). This protein, which we have designated as NF-βA (24), binds to a conserved sequence (ACTTCTGCTTTT) found at the identical location in the human and mouse genes (−49 to −38) and is expressed constitutively in competent cells but is not expressed in refractory cells such as T cells and HeLa cells. It is not yet clear that NF-βA is responsible for the observed tissue specificity, since a recognition sequence for a factor with appropriate tissue specificity, the hematopoietic transcription factor Spi-1 (equivalent to factor B1 in [19]), also is contained within the 131 residues of upstream promoter-proximal sequence as well as a recently reported binding site for NF-IL6 (35). We have recently demonstrated an involvement of this part of the proIL-1β gene sequence in the *trans* activation of this gene by the IE1 immediate-early gene product of human cytomegalovirus (HCMV), thus explaining the strong effect of HCMV infection on the induction of IL-1 (26). The fact that HCMV IE1 *trans* activation is enhanced in the presence of LPS (10) suggests an additional complexity to the nature of proIL-1β gene regulation.

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